Absence of neuronal autoantibodies in neuropsychiatric systemic lupus erythematosus

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Format: Brief communication

Word count: 1548

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ana.25908

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Abstract

This study aimed to characterise both neuronal autoantibodies and levels of IFNα, two proposed causative agents in neuropsychiatric systemic lupus erythematosus (NPSLE). CSF and plasma from 35 SLE patients (15 with NPSLE) showed no antibodies against natively-expressed NMDA receptors, or the surface of live hippocampal neurons. By comparison to controls (n=104), SLE patient antibodies bound a peptide representing the extracellular domain of NMDARs ($p<0.0001$), however, binding was retained against rearranged peptides and no peptide ($r=0.85$ and $r=0.79$, respectively, $p<0.0001$). IFNα levels were higher in SLE ($p<0.0001$), without specificity for NPSLE. Neuronal-surface reactive autoantibodies are not detectable in NPSLE. Our findings mandate a search for novel biomarkers in this condition.

Introduction

Systemic Lupus Erythematosus (SLE) is a multi-system autoimmune disease associated with antibodies against native DNA. A subset of patients have neuropsychiatric SLE (NPSLE), a disabling clinical syndrome with widely varied features including headache, seizures, stroke, and psychosis.[1,2] This clinical heterogeneity means accurate biomarkers are required to better define NPSLE.

Human autoantibodies are one proposed biomarker. This field began with characterisation of a double-stranded DNA (dsDNA) reactive mouse monoclonal antibody (R4A). Upon transfer to mice, R4A led to glomerular deposition, a key feature of SLE pathology. Importantly, a peptide phage display library revealed R4A bound a consensus pentapeptide sequence (D/E-W-D/E-Y-S/G, DWEYS for short) present within the extracellular domain of the NR2A and NR2B subunits of the N-methyl-D-aspartate receptor (NMDAR), and induced neuronal death in mice.[3,4] Also, DWEYS-reactive B cell receptors isolated from SLE patients, including the G11 monoclonal antibody, were reported to bind NMDARs.[5] These observations led to the proposal of NMDAR-directed autoantibodies as pathogenic agents in NPSLE.

Yet, human autoantibodies against NR2A/B subunit peptides have been disputed as a clinically useful test for NPSLE.[6-8] A recent meta-analysis of ~3000 subjects observed these serum autoantibodies in more patients with

SLE and Sjögren's syndrome versus controls, but with only a modest increase in the neuropsychiatric subset.[8] Very few studies presented parallel data from cerebrospinal fluid (CSF).

An independent literature describes a clinically-distinctive form of encephalitis, with prominent neuropsychiatric features, which is consistently associated with serum and CSF autoantibodies against the native extracellular domain of the NMDAR NR1 subunit.[9,10] These patients, with NMDAR-antibody encephalitis, very rarely have coexistent SLE, despite often being young females.[9,10] Their NR1-reactive autoantibodies also bind the surface of live hippocampal neurons but not NR1-derived peptides, consistent with selectivity for native epitope confirmations.[9-11] Similar exclusive binding to native, conformational surface proteins exists for other pathogenic neurological antibodies, directed against leucine-rich glioma inactivated 1 (LGI1), contactin-associated protein-like 2 (CASPR2) and aquaporin-4 (AQP4).[12,13]

Herein, we show that IgGs from sera and CSF of NPSLE patients neither react with surface-expressed native neuronal epitopes, nor show specificity towards peptides representing NMDARs. Our data support a fundamental need to redirect the search for biomarkers in human NPSLE, away from causative autoantibodies.

Patients and Methods

Patient selection and involvement

Paired plasma and CSF samples were frozen from 35 consecutive patients who met American College of Rheumatology (ACR) criteria for SLE, in a tertiary neuroinflammation clinic (Karolinska University Hospital, Sweden). Clinical and laboratory features were extracted retrospectively from case-notes. Control plasma and CSFs were from healthy controls (HC, n=36), with additional plasmas from CNS-autoimmunity disease controls (DC, n=68: multiple sclerosis (n=32) plus LGI1/CASPR2- (n=13), AQP4- (n=12) and NMDAR- (n=11) antibody mediated diseases). Study procedures and sample testing were ethically approved (Stockholm:2009/2107-31/2; Oxford:REC16/YH/0013). All assays were performed blinded to clinical details.

Autoantibody determination and IFNα measurements

As previously described,[10,14] plasma (1:20 dilution) and CSF (1:2 dilution) underwent live cell based assays (CBA) to detect autoantibodies against the extracellular domains of conformationally active NMDARs. Also, plasma (1:200) and CSF (1:2) were incubated with live cultured rodent hippocampal neurons after around day 18 *in vitro*. The DWEYS ELISA followed a published protocol,[4] coating plates with either 20 μg/ml of DWEYSVWLSN ('DWEYS'), a decapeptide with two substitutions (KWRYSVWLSN; with positively charged amino acids replacing negative ones), a randomly scrambled DWEYS

decapeptide (SDYVWLNESW) or no peptide. Plasma (1:500), CSF (1:1) and the humanised DWEYS monoclonal antibody (G11, kind gift from Prof Diamond, USA) were incubated for 1 hour prior to washing and incubation with anti-human/mouse HRP (1:2000 dilution, DAKO) followed by TMB (Bethyl) to develop the reaction. IFNα quantification utilised single molecule array (SIMOA) technology, as previously reported.[15]

Results

Patient and CSF characteristics

At the time of assessment and sampling, median patient age was 47 years (range 18-77), 34/35 (97%) were female and 32/34 (94%) were receiving immunotherapies. 23/35 had active SLE and 15 active NPSLE, with multiple neurological features (Figure 1A).

CSFs revealed raised CSF IgG in 11/35 (31%, normal range <45 mg/L), with accompanying raised IgG index in just one, and pleocytosis in 5/35 (14%, normal range <5 cells/μl). In addition, 12/35 (34%) showed a raised albumin quotient (normal range <7.1), consistent with blood-brain barrier dysfunction. No differences in these parameters were observed between subjects with and without NPSLE.

Absent binding of patient plasma/CSF IgGs to the extracellular domains of either expressed NMDARs or live neuronal cultures

From live CBAs, surface expression of NR1/2 subunits was confirmed using commercial antibodies and the serum/CSF IgGs from NMDAR-antibody encephalitis patients (Figure 1B-C).[9,10] However, the 35 SLE serum/CSF samples, HC sera and the G11 monoclonal antibody neither showed binding to the extracellular domains of mammalian cell expressed NR1/2A or NR1/2B heteromers nor to the array of native surface proteins expressed on live rodent primary hippocampal cultures (Figure 1B). This contrasted strikingly with the strong punctate somatic and dendritic binding observed with plasma/CSF IgGs from patients with encephalitis, including those with NMDAR- and CASPR2-antibodies (Figure 1D).[9,12]

Autoantibody binding to NMDAR-derived peptides is not peptide-specific

Next, the original detection of DWEYS antibodies was revisited. On DWEYS-coated ELISA plates, the anti-DWEYS G11 monoclonal bound clearly and an isotype control (B1) consistently showed ODs <0.3, both as predicted (Figure 2A). Statistically significant differences were observed between SLE patients versus both HCs ($p=0.03$) and DCs ($p=0.009$). However, using a conservative cut-off, plasma IgG from 8/35 SLE patients, 2/36 HCs and 1/68 DC showed DWEYS reactivity (Figure 2A), and only 3/35 SLE samples, including one without active NPSLE, were clearly above all HC/DCs (Figure 2A). This

translated to ~23% sensitivity and ~97% specificity for detection of SLE, and did not distinguish the NPSLE cases. Similarly, CSF DWEYS reactivity did not discriminate between SLE and HCs, with a modest difference between active NPSLE and other SLE cases (p=0.021, Mann Whitney U; Figure 2B). Overall, the DWEYS IgG ELISA showed very limited specificity for NPSLE but cohort-based differences revealed higher levels in SLE versus HC/DC plasmas.

Next, this antigenic specificity was interrogated. Individual plasma samples showed very similar binding to the DWEYS peptide and to both the scrambled peptide 'SDYVW' (r=0.73, p<0.0001) and a substituted peptide 'KWRYS' (r=0.72, p<0.0001; Figure 2C-D). Furthermore, the DWEYS-reactivity from plasma and CSF correlated with binding to the uncoated ELISA plate (r=0.85, p<0.0001 and r=0.79, p<0.0001, respectively; Figure 2E).

IFNα levels better distinguish SLE from controls, but not from NPSLE

Plasma IFNα levels were investigated as IFNα is associated with SLE, the development of neurological autoantibody-mediated complications in SLE, and is directly a neurotoxic cytokine.[15-17] Plasma IFNα levels were ~100-fold higher in patients with SLE compared to HCs/DCs (both p<0.0001; Figure 3A), with less striking differences in CSF (Figure 3B). Consistent with their close correlations (Figure 3C), neither fluid distinguished active NPSLE from other SLE cases (Figure 3A-B). Using mean plus three standard deviations of HC

data as a cut-off for IFNα 'positivity', 22/35 (63%) SLE patients, 1/36 (3%) HC and 1/68 (1%) DC were positive in plasma, and 8/35 (23%) SLE patients and 1/36 (1%) HC in CSF (sensitivities and specificities of ~63% and ~97% in plasma, and ~23% and ~99% in CSF).

Discussion

Our results show the absence of autoantibodies directed against conformationally-active neuronal surface targets, in both the plasma and CSF of patients with NPSLE. Furthermore, while SLE samples reacted more strongly with the DWEYS peptide, this binding appears to be accounted for by reactivity directly to the ELISA plate, suggesting a lack of antigenic specificity. The use of suitable antigen controls is therefore key in NPSLE studies, and may account for conflicting results in previous human reports. Taken together, our data suggest that autoantibodies have very limited clinical value in the definition or diagnosis of NPSLE and should prompt a search for novel biomarkers. IFNα is one such plausible candidate.[15,16] However, while IFNα levels performed slightly better in distinguishing SLE from controls, they were also not enriched in the NPSLE subset.

In routine neurology practice, autoantibodies which bind the surface of membrane proteins, such as the NMDAR, typically associate closely with a highly-distinctive clinical phenotype.[9,10,12] These autoantibodies preferentially

react with conformationally-active, natively-expressed forms of the whole antigen: this confers pathogenic potential.[9-13] However, we could not detect autoantibodies which react with the natural range of surface epitopes presented on cultured hippocampal neurons or, more specifically, the extracellular domain of native NMDARs in patients with SLE or NPSLE,[18] despite proven adequate surface expression.

Several observations question the clinical and pathogenic relevance of DWEYS-peptide reactive antibodies.[7,8,19] Firstly, the DWEYS motif exists in multiple other human proteins, such as caeruloplasmin. Next, binding of a monoclonal antibody to a small peptide, without reacting with the full-length, conformational protein, is unlikely to mediate target-specific functional effects *in vivo*. Thirdly, DWEYS-reactive IgGs showed similar binding to scrambled and no peptide conditions. Hence, these antibodies do not specifically bind DWEYS. Rather, the physiochemical properties of naturally occurring self-reactive antibodies, which are well described in SLE, are likely to account for their promiscuous binding characteristics.[20]

Limitations of this study include a moderate cohort size and no examination of native glial or vascular reactivities, where the latter in particular may account for complement deposition observed in NPSLE brain tissue.[21] In addition, we study only one cytokine and use rodent hippocampal neurons which in culture

may not to express key surface antigens for human NPSLE. Nevertheless, our use of parallel assays has been successful in the detection of >10 other likely pathogenic neurological autoantibodies previously,[12,13] we test a sizeable set of CSFs and employ rigorous control peptides which accurately assess autoantibody specificity.

Our observations suggest that the brain disease associated with SLE is likely to have pathological drivers other than neuronal surface autoantibodies. Future studies might take broader 'omics' approaches to assessing this clinically important manifestation of SLE, investigate innate immune components in greater detail and identify reliable biomarkers for diagnosis, monitoring and to better understand the primary pathology.

Acknowledgements

JV was supported by the Association of British Neurologists and the Guarantors of Brain. SRI is supported by the Wellcome Trust (104079/Z/14/Z), The UCB-Oxford University Alliance, BMA Research Grants – 2013 Vera Down grant and 2017 Margaret Temple, Epilepsy Research UK (P1201) and by the Fulbright UK-US commission (MS-Research Society Award). PW is supported by the UK NMO commissioning group. The work is supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC). The views expressed are those of the authors and not necessarily those of the NHS,

the NIHR, or the Department of Health. DD acknowledges support from the ANR (CE17001002) and thanks Immunoqure for provision of mAbs for the SIMOA assay.

Author contributions

JV, FP, PW and SRI contributed to the conception and design of the study; JV, MA, EG, AB, MZ, VB, DD, DH, FP, PW and SRI contributed to the acquisition and analysis of data; JV, EG, AB, DD, DH, PW and SRI contributed to drafting the text and preparing the figures.

Potential conflicts of interest

Nothing to report

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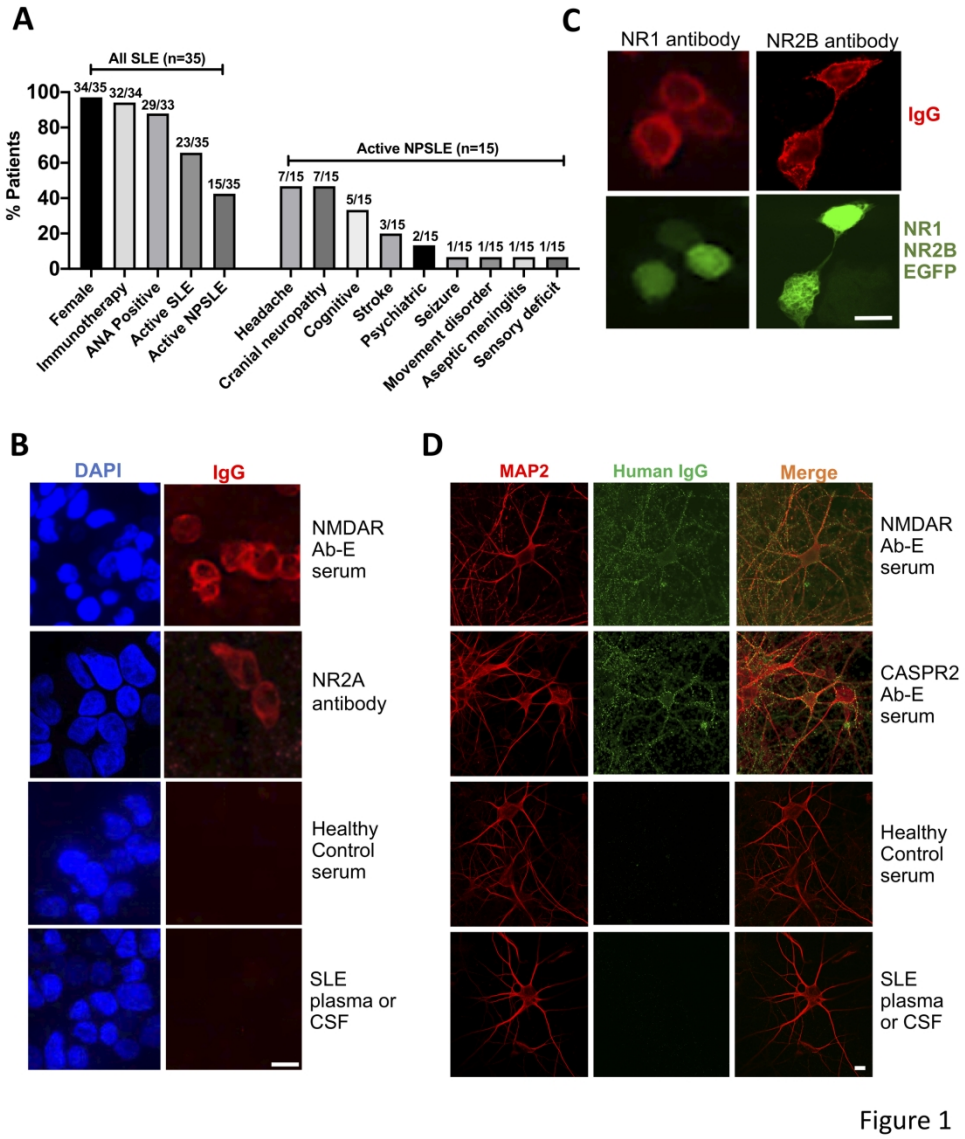
Figure legends

Figure 1. **Clinical features and immunoglobulin G (IgG) binding to conformational neuronal surface epitopes from plasma and cerebrospinal fluid (CSF) of patients with neuropsychiatric lupus (NPSLE). A.** Clinical features of 35 patients with SLE, including 15 with active NPSLE at the time of sampling. 32/34 (94%) were on immunotherapies, including prednisolone (71%), hydroxychloroquine (41%), belimumab (6%) and rituximab (3%). ANA = anti-nuclear antibody. **B.** Live cell-based assays employing HEK293T cells with surface expressed N-methyl D-aspartate receptors (NMDARs) as NR1-NR2A

or NR1-NR2B heteromers (nuclei highlighted with DAPI, 4′,6-diamidino-2-phenylindole). First panel shows that a commercial antibody directed against the extracellular domain of the NR2A-subunit (red) binds to HEK293T cells which express NR1-NR2A heteromers. NMDAR-antibody encephalitis (NMDAR-Ab-E) patient serum IgGs (n=11,) bind to the NR1-NR2A heteromers (second panel), and to NR1-NR2B heteromers / NR1 homomers (data not shown). NMDAR-antibody patient CSFs show similar binding (as Irani et al, 2010).[10] Serum from healthy controls (n=36), plasma and CSFs from SLE patients (n=35 of each) show no binding. No binding was observed with the G11 antibody (data not shown, lower panel representative). Throughout, IgG binding was visualized with an anti-human 568-Alexaflour antibody (1:750 dilution, A-21090) **C.** Commercial antibodies against the extracellular domains of NR1 (Alomone, AGC-001), NR2A (Alomone, AGC-002) and NR2B (kind gift from Prof Stephenson, London) subunits bound to the surface of live HEK293T cells co-transfected with EGFP plus NR1 and NR2B subunits. **D.** Hippocampal neuron somae and dendrites are stained with a commercial antibody against microtubule associated protein 2 (MAP2, red). Live neurons in culture are robustly bound by IgGs from sera (top two panels) or CSF (data not shown) of patients with NMDAR-antibody encephalitis (n=11) and CASPR2-antibody encephalitis (n=5; CASPR2-Ab-E). However, no binding was detected using plasma / CSFs from 35 SLE patients and 36 sera from healthy controls. All scale bars =10 µm.

Figure 2. Plasma and cerebrospinal fluid (CSF) reactivities against DWEYS and related peptides. A. Optical density (OD) results from the DWEYS ELISA, using G11 (humanized anti-DWEYS antibody), an isotype control (B1), and plasma from disease controls (n=68), healthy controls (n=36) and SLE patients (n=35), including those with active NPSLE. Significant differences (Kruskal Wallis test with post-hoc Dunn's correction) were observed between SLE patients and healthy controls (p=0.03*) and disease controls (p=0.009**), but not between active NPSLE and other cases of SLE (ns). Cut-off for positivity (dotted line) represents the mean plus three standard deviations of healthy control values. **B.** From CSF, the DWEYS ELISA showed no difference between HCs and SLE, and modest difference within a comparison of SLE patients with active NPSLE versus others (p=0.021, Mann Whitney U test without correction for multiple comparisons). **C and D.** ELISA findings across all 35 SLE patients paralleled each other when using the DWEYS peptide, a scrambled peptide (SDYVWLNESW; Spearman's r 0.72, p<0.0001) and a peptide with two positive amino acid substitutions (KWRYSVWLSN; Spearman's r 0.73, p<0.0001). **E.** In both plasma (black) and CSF (grey, data multiplied ten fold), the ODs were highly correlated when performed in the presence versus absence of DWEYS peptide (Spearman's rank r=0.85 and 0.79, respectively, both <0.0001).

Figure 3. Interferon alpha (IFNα) levels in neuropsychiatric systemic lupus erythematosus (NPSLE). A. Plasma IFNα levels differentiate SLE from healthy controls (n=20) and from disease controls (n=32; both p<0.0001****, Kruskal Wallis with post hoc Dunn's). There were no differences between SLE patients with active NPSLE versus those without (p=0.39). Comparisons of active NPSLE and other SLE versus both HC or DC all p<0.01 (data not charted). **B.** CSF IFNα does not differentiate between SLE and HC but does SLE and DC (p=0.02, Kruskal Wallis with post hoc Dunn's). There was no difference between active NPSLE versus other SLE. IFNα levels in active NPSLE versus DC are significantly different (p=0.03) but not by comparison to HC. **C.** Overall, plasma and cerebrospinal fluid IFNα levels correlate closely (Spearman's r=0.68, p< 0.0001) in both active NPSLE (r=0.75, p=0.002) and inactive NPSLE (r=0.59, p=0.007).



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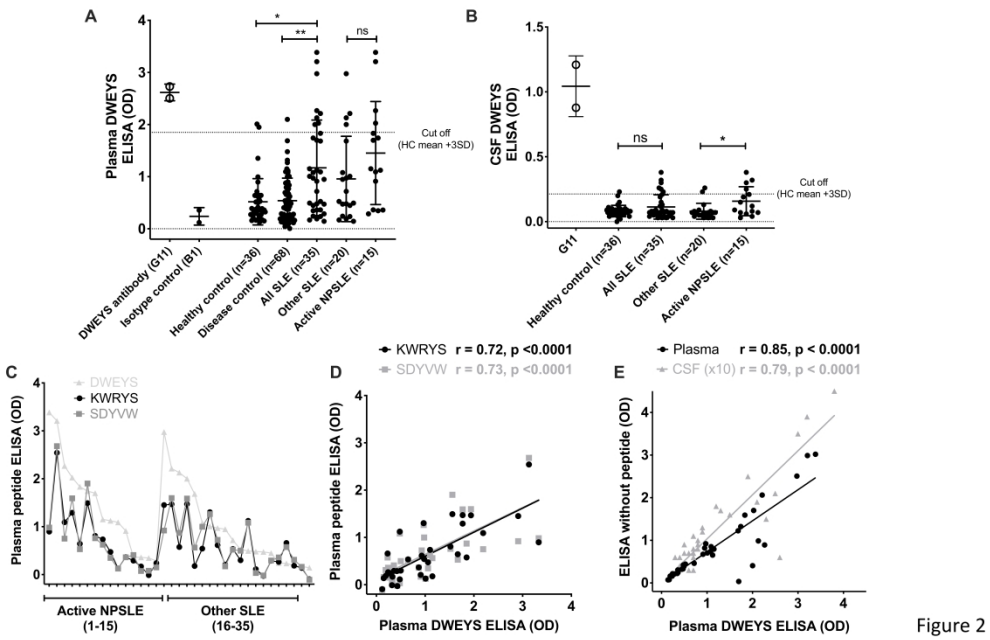

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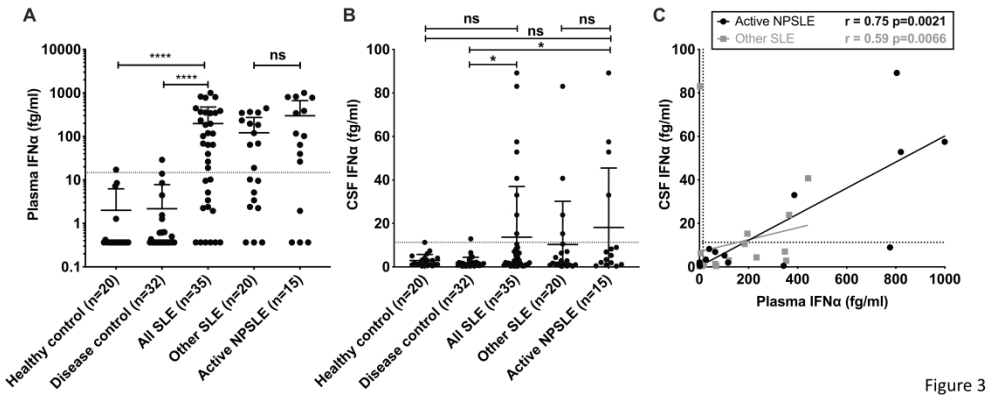

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